Bacterial Dissemination to the Brain in Sepsis

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Abstract

Rationale: Sepsis causes brain dysfunction and neuroinflammation. It is unknown whether neuroinflammation in sepsis is initiated by dissemination of bacteria to the brain and sustained by persistent infection, or whether neuroinflammation is a sterile process resulting solely from circulating inflammatory mediators.

Objectives: To determine if gut bacteria translocate to the brain during sepsis, and are associated with neuroinflammation.

Methods: Murine sepsis was induced using cecal ligation and puncture, and sepsis survivor mice were compared with sham and unoperated control animals. Brain tissue of patients who died of sepsis was compared with patients who died of noninfectious causes. Bacterial taxa were characterized by 16S ribosomal RNA gene sequencing in both murine and human brain specimens; compared among sepsis and nonsepsis groups; and correlated with levels of S100A8, a marker of neuroinflammation using permutational multivariate ANOVA.

Measurements and Main Results: Viable gut-associated bacteria were enriched in the brains of mice 5 days after surviving abdominal sepsis ($P < 0.01$), and undetectable by 14 days. The community structure of brain-associated bacteria correlated with severity of neuroinflammation ($P < 0.001$). Furthermore, bacterial taxa detected in brains of humans who die of sepsis were distinct from those who died of noninfectious causes ($P < 0.001$) and correlated with S100A8/A9 expression ($P < 0.05$).

Conclusions: Although bacterial translocation is associated with acute neuroinflammation in murine sepsis, bacterial translocation did not result in chronic cerebral infection. Postmortem analysis of patients who die of sepsis suggests a role for bacteria in acute brain dysfunction in sepsis. Further work is needed to determine if modifying gut-associated bacterial communities modulates brain dysfunction after sepsis.

Keywords: inflammation; microbiome; S100A8; sepsis; cecal ligation and puncture

Sepsis, a syndrome of life-threatening organ dysfunction associated with the host response to infection, is frequently associated with acute and chronic brain

dysfunction (1–5). Patients who die of sepsis experience cerebral coagulopathy, ischemia, and inflammation, and in animal models sepsis results in persistent

neuroinflammation. Although multiple mechanisms related to the host response to infection provoke end-organ injury in sepsis, it is unclear whether the systemic

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At a Glance Commentary

Scientific Knowledge on the

Subject: Sepsis results in long-term brain dysfunction, and animal models indicate that neuroinflammation plays a role in brain injury during sepsis. Whether brain injury is related to direct translocation of bacteria to the brain and cerebral microvasculature, or is a result of sterile inflammatory mediators is unknown. Evidence from patients with sepsis and animal models of sepsis and pneumonia suggest that translocation of gut bacteria may potentiate lung inflammation during sepsis, but the role of bacterial translocation in other organs is unstudied.

What This Study Adds to the

Field: In experimental sepsis, early polymicrobial dissemination of bacteria to the brain is followed by persistence of a limited number of aerobic and anaerobic bacterial taxa that are eventually cleared, even in the absence of bacteremia. In postmortem brain specimens, the bacterial taxa identified from patients who die of sepsis are distinct from those of patients who died of noninfectious causes. The presence of bacteria is associated with an elevated neuroinflammatory marker in both mice and patients. We conclude that sepsis-related brain dysfunction is not a state of chronic bacterial encephalitis but that bacteria are associated with the early brain response to sepsis.

dissemination of gut microbiota is an important part of the development of organ dysfunction, or whether organ dysfunction is solely caused by a dysregulated, but mostly sterile, host response (6–9). We have previously found that sepsis and acute respiratory distress syndrome are associated with enrichment of gut-associated bacteria in the lungs (10). Dissemination of enteric bacteria is not unique to models of abdominal sepsis; *Pseudomonas* pneumonia results in dissemination of enteric bacteria (11). It is therefore plausible that viable bacteria, and not only circulating cytokines, stimulate inflammation in organs remote from the initial site of infection.

Systemic administration of LPS and other purified bacterial products is often used as a model of sepsis and activation of innate immunity (12). Direct and overwhelming agonism of the signaling pathways downstream of LPS does produce significant neuroinflammation, including morphologic changes and cytokine expression in microglia, the primary innate immune cell of the brain (13). However, growing evidence indicates that even in sterile inflammation, the dose and timing of exposure to inflammatory mediators can lead either to priming or suppression of innate immunity (14). The presence over time of whole bacteria and multiple bacterial antigens within the microcirculation or the brain parenchyma, then, may provoke a significantly different immune response than a single large exposure to specific pathogen-associated molecules.

We hypothesize that neuroinflammation in sepsis and in sepsis survivors is not only provoked by sterile inflammatory mediators, but is related to the presence of bacteria in the brain. We further hypothesize that bacterial translocation is transient, and that the prolonged neuroinflammation observed in animal models of sepsis survival is not simply related to unresolved infection (9, 15). Here, we have used a naturalistic murine model of sepsis to show that viable gut-associated bacteria disseminate to the brain and are found for days after infection even in sepsis-survivor mice. We bolster the plausibility of bacterial dissemination as a driver of the brain response to infection with evidence of bacterial dissemination to the brain associated with neuroinflammation in humans who died of sepsis, even in the absence of clinically notable bacteremia. Some of the results of these studies have been previously reported in abstract form (16).

Methods

Mice

Wild-type mice used in these experiments were male C57BL/6 mice obtained from Jackson Laboratory and used at 8–10 weeks of age. CX3CR1-GFP::CCR2-RFP reporter mice were used at 8–12 weeks of age (17).

Sepsis Model

Cecal ligation and puncture (CLP) was performed as previously described using a single puncture with a 19-gauge needle (18). Imipenem/cilastatin (Merck, 0.5 mg/mouse) and normal saline (1 ml) were administered subcutaneously at the time of surgery. Sham operated animals underwent laparotomy without ligation or puncture of the cecum, and received antibiotics and saline.

Colony-Forming Unit Assay

Aliquots of brain homogenates, blood, or peritoneal lavage fluid were plated onto LB media in 10-fold dilutions and incubated aerobically at 37°C. Colonies were counted after 24 hours of growth and identified through a colony PCR method (10, 19).

Identification and Isolation of Patient-derived Brain Tissue

Human brain specimens were identified from the University of Michigan Brain Bank. Patients were identified as having sepsis if they died with a diagnosis of sepsis or had evidence of infection and organ injury. Patients were identified as dying of noninfectious causes if they died without evidence of infection and with an identifiable cause of death. These cases have also been examined in a separate publication examining the expression patterns of S100A8 in sepsis.

Bacterial DNA Isolation and 16S DNA Sequencing

Genomic DNA was extracted from mouse or human brain tissue. The V4 region of the 16S ribosomal RNA (rRNA) gene was amplified using published primers (20) and the dual-indexing sequencing strategy developed by the laboratory of Patrick D. Schloss and as previously described (10, 21).

Viability Assay of OTU008

Sequences matching the Bacteroidales OTU (OTU008, 100% coverage and identity) were identified using NCBI BLAST and have been previously identified in mouse feces and colonic mucosa (22–24). To determine whether OTU008 represented a viable bacterium or residual nucleic acids from killed organisms, we cultured brain homogenates under anaerobic conditions. PCR using OTU008-specific primers was performed to verify the presence

Figure 1. Multiple bacterial taxa are detectable in the brain during early sepsis. Abdominal sepsis was induced in mice by cecal ligation and puncture, and bacterial DNA detected in homogenized brain tissue was studied after 24 hours. (A) Brain bacterial communities 24 hours after operation are distinct from unoperated control subjects in both sham-operated and septic animals (permutational multivariate ANOVA, $P = 0.03$), and all three groups are distinct by principal component analysis ($P < 0.001$ for all pairwise comparisons). (B) Multiple bacterial taxa are detected in brain homogenates 24 hours after induction of sepsis, without clear predominance of a single taxon. These taxa are generally not represented in the sequences recovered from either sham-operated or unoperated animals. Gray bars indicate background signal detected in brains of unoperated mice. Data presented are means $\pm$ SEM. CLP = cecal ligation and puncture; Otu = operational taxonomic unit; PC = principal component.

of reproducing bacteria in these mixed cultures.

Statistical Analyses

Sequence data were processed and analyzed using the software *mothur* version 1.27.0, as previously described (25). Comparisons among means or medians were performed as noted in the text. Detailed methods are presented in the online supplement.

Results

Sepsis Induces Transient Dissemination of Bacteria to the Brain

To study whether sepsis results in dissemination of bacteria to the brain, we used CLP, a well-characterized murine model of polymicrobial sepsis and survivorship (9, 18). We have previously shown that CLP results in leukocyte infiltration and inflammatory cytokine production in the brain for at least 2 weeks after sepsis, with recovery of leukocyte infiltration but sustained cytokine

expression over the following 50 days (9). We therefore studied microbiota isolated from the brain and blood via both culture and sequencing of 16S ribosomal RNA-encoding genes with the primary goal of determining whether persistent neuroinflammation after sepsis is simply a result of low-grade chronic bacterial encephalitis.

Given that CLP has the potential to disseminate aerobic and anaerobic bacteria, we performed an unbiased analysis of bacterial taxa present in the brain by 16S rRNA gene sequencing. One day after the induction of sepsis, bacterial sequences detected in the brains of septic mice were distinct from sham-operated and unoperated control animals (Figure 1A) (permutational multivariate ANOVA, $P = 0.008$). Unoperated control animals did not receive antibiotics. The differences among treatment groups reflects a wide array of bacterial taxa that differ from the background signal detected in unoperated control animals and reflect expected

members of the murine gut microbiota, without clear predominance of a single taxon (Figure 1B). No bacteria grew in aerobic culture of the blood 24 hours after CLP, sham operation, or in unoperated control animals ($n = 4$ per group, not shown).

To determine whether CLP results in prolonged exposure of the brain to bacteria, we examined brain microbiota in sepsis-survivor mice. Five days after intervention, bacterial sequences detected in brains of sepsis-survivor mice were distinct from those derived from sham-operated, antibiotic-treated, and untreated animals ($P = 0.002$) (Figure 2A). By 14 and 50 days after CLP bacterial sequences were not distinct among groups ($P = 0.498$ and 0.532) (see Figure E1C in the online supplement). In contrast to acutely ill animals, differences in bacterial community structure at 5 days after sepsis were primarily driven by a single Bacteroidales-classified taxonomic group (OTU), OTU008 followed by an *Enterococcus* classified OTU (Figure 2B). The relative

Figure 2. Brain microbial communities evolve after cecal ligation and puncture. Sepsis was induced by cecal ligation and puncture, and bacterial DNA detected in homogenized brain tissue was studied in surviving mice and compared with sham-operated, antibiotic-treated, and untreated control animals after 5 days. (A) Brain bacterial communities 5 days after induction of sepsis are distinct from those isolated from all control groups (permutational multivariate ANOVA, $P < 0.002$). (B) By 5 days post-cecal ligation and puncture, the detected microbial taxa are dominated by Bacteroidales OTU008, which is not detected in control groups. (C) Bacteroidales OTU008 dominates the bacterial community 5 days after sepsis ($**P < 0.01$) and returns to background levels by 14 days. Gray bars indicate background signal detected in brains of unoperated mice. Values presented as means $\pm$ SEM. Statistical significance was determined by Kruskal-Wallis one-way ANOVA with Dunn multiple comparison test (C). OTU = operational taxonomic unit; PC = principal component.

abundance of this Bacteroidales OTU was significantly increased in sepsis-survivor animals compared with all control mice at 5 days after sepsis ($P \leq 0.01$), but returned to background levels by 14 days (Figure 1C).

To determine whether the sequences detected by 16S rRNA gene analysis represented viable bacteria or residual nucleic acids from killed organisms, we cultured brain homogenates from sepsis-survivor mice. *Enterococcus faecalis* was isolated from aerobic culture of brains of sepsis-survivor mice 5 days after CLP (Figure 3A), but was not cultured from the blood (see Figure E1A).

E. faecalis was not cultured from the brain 14 days or 50 days after CLP, or from sham-operated animals or antibiotic-treated unoperated control animals (Figure 1A; see Figure E1B). To examine the viability of OTU008, we cultured brain homogenates 5 days after CLP and from unoperated control mice under anaerobic conditions. OTU008 was undetectable by PCR of raw brain lysates of sepsis-survivor or control mice, but was readily amplified from anaerobic cultures of brain tissue, indicating that viable bacteria grew *in vitro* and are thus present in

the brains of sepsis-survivor mice (Figure 3B).

Neuroinflammation Is Associated with Altered Brain Microbial Communities in Patients with Sepsis and in Experimental Sepsis

Given this evidence of significant bacterial exposure in the brain in our experimental model of sepsis, we questioned whether brain microbial communities in patients with sepsis differ from those in patients who died of nonsepsis causes. We reviewed records of subjects in the University of Michigan Brain Bank

Figure 3. Experimental sepsis results in translocation of live aerobic and anaerobic bacteria to the brain. (A) Five days after cecal ligation and puncture, *Enterococcus faecalis* was isolated from the brains of five of six postsepsis mice but was undetectable in the brains of mice in all control groups ($P = 0.002$). (B) OTU008 corresponds to a live bacterium, not carryover of nucleic acids. PCR specific to OTU008 shows the presence of bacterial growth in brain homogenates in anoxic conditions after sepsis but fails to detect OTU008 signal in raw brain homogenates or in unoperated control animals. Values presented as means $\pm$ SEM. Statistical significance was determined by Kruskal-Wallis one-way ANOVA with Dunn multiple comparison test (A). OTU = operational taxonomic unit.

who had died either of sepsis (see Table E1) or of a clear noninfectious cause (see Table E2).

We found marked variability in the microbiota detected in the brains of patients with sepsis, including high abundance of taxa associated with both the upper respiratory and lower gastrointestinal tracts

(Figure 4A). The separation between sepsis and nonsepsis brain specimens as a group was driven primarily by differences in relative abundance of *Haemophilus*, *Neisseria*, and *Moraxella* species, with brain specimens of individual patients with sepsis dominated by gut-associated taxa, such as *Enterobacteriaceae* sp. and *Bacteroides* sp.

None of the sepsis-associated microbial taxa were enriched in the negative sequencing control specimens. Overall, bacterial taxa identified by 16S rRNA gene sequencing in cerebral cortex tissue were distinct in patients who died of sepsis compared with nonsepsis control subjects (PERMANOVA, $P = 0.0008$).

Case reports have suggested that patients who die of sepsis demonstrate increased brain cytokine expression associated with neuroinflammation (26). We therefore examined expression of IL-1 α , IL-1 β , IL-1RA, IL-4, IL-6, IL-8, CCL2, CCL3, CCL4, and tumor necrosis factor- α in brain lysates by multiplex assay (Luminex, R&D Systems). Once adjusted for multiple comparisons, there was no significant difference in the expression of any of these inflammatory mediators in the brains of patients who died of sepsis and noninfectious causes (see Figure E2). Based on prior animal studies, we hypothesized that expression of the endogenous inflammatory mediator S100A8/A9 would be elevated in patients with sepsis (9).

Using S100A8/A9 protein concentration as a covariate in analysis of 16S sequences identified from human brain specimens, we found that neuroinflammation as measured by S100A8/A9 protein concentration was significantly correlated with the community structure of bacterial taxa (PERMANOVA, $P = 0.034$) (Figure 4B), indicating that human brain specimens with similar concentrations of S100A8/A9 had similar bacterial community structure as measured by 16S rRNA gene sequencing.

S100A8 expression is associated with brain microbiota even in early survivors of murine experimental sepsis. We measured brain expression of S100A8 in cerebral cortex tissue from the same animals used to study changes in microbiota. S100A8 gene expression was markedly increased in the cerebral cortex 5 days after sepsis compared with control conditions ($P \leq 0.001$) (Figure 5A). S100A8 expression was significantly associated with the community structure of bacterial taxa in the brains of mice, driven by differences in the relative abundance of the previously described *Bacteroidales* OTU008 ($P \leq 0.001$) (Figure 5B). Sepsis survivor mice also display evidence of altered microglial responses 5 days after CLP. Immunoreactivity for the microglial marker Iba1 was increased in the hippocampus

Figure 4. Neuroinflammation is associated with altered brain microbiota in patients with sepsis. Bacterial DNA was isolated and sequenced from postmortem brain specimens in patients who died of sepsis or a noninfectious illness. (A) Heterogeneous bacterial taxa are detected in brains of patients with and without sepsis. Model-based analysis of multivariate abundance identified *Haemophilus*, *Neisseria*, and *Moraxella* spp. as differentially abundant in sepsis and nonsepsis brain communities. A single brain specimen was enriched with *Enterobacteriaceae* sp. (OTU0017) and two were enriched with *Bacteroides* sp. (OTU0014). Pink boxes indicate upper airway-associated taxa, and brown boxes indicate gut-associated taxa. (B) Bacterial communities detected in cerebral cortex specimens from patients with sepsis were distinct from those of patients who died of noninfectious illness ($P < 0.001$). S100A8/A9 (S100 calcium binding protein A8 and A9 heterodimer) concentration is correlated with differences in bacterial taxa ($P < 0.05$). The area of each circle is proportional to the level of S100A8/A9 protein. Statistical significance was determined by Mann-Whitney U test (A) and permutational multivariate ANOVA (*adonis*) (B). OTU = operational taxonomic unit; PC = principal component.

and motor cortex compared with sham-operated animals (Mann-Whitney U test; $P = 0.035$) (Figures 5C and 5D).

Isolated microglia and tissue macrophages, identified using a GFP reporter of CX3CR1 expression (Figure 5E), demonstrate priming of tumor necrosis factor- α production on *ex vivo* stimulation with LPS (Figure 5F). Neuroinflammation in sepsis survivor mice, which we have previously characterized up to 2 months following CLP (9), could simply be driven by chronic intraperitoneal infection and systemic inflammation. However, peritoneal infection (*see* Figure E3A) and neutrophil exudation (*see* Figure E3B) both improve significantly over the first week following CLP.

Discussion

The central finding of our study is that sepsis results in dissemination of bacteria to the brain for several days, which ultimately resolves over the course of weeks after the onset of illness. The presence of distinct bacterial taxa was associated with S100A8 expression, a marker of neuroinflammation, both in animals and humans who died of sepsis. Our results suggest that viable bacteria, and not only

sterile circulating inflammatory mediators, play a role in the pathogenesis of brain dysfunction after sepsis.

The brain is vulnerable to numerous insults during sepsis. Cerebral infarcts, hemorrhage, and microabscesses and macroabscesses have been demonstrated in postmortem studies of patients who die of sepsis (27–29). One could assume that any patient with bacteremia is at risk for developing microabscesses in multiple organ systems, and that this process underlies organ dysfunction in sepsis. Some common bacteria, such as *Staphylococcus aureus*, do form disseminated abscesses in the case of fulminant infection, including in the brain (30). Likewise, direct dissemination of *Streptococcus* to the myocardium contributes to cardiac dysfunction in models of pneumonia (31). Although the prevalence of focal infection at autopsy is common in sepsis, disseminated microabscesses are rare, and encephalitis or brain abscesses were nearly absent in several case series (32–34). Similarly, in the present study only a single patient had gross or histopathologic evidence of overt brain infection at autopsy (*see* Table E1).

If histologic evidence of bacterial dissemination is absent in many cases of sepsis, then is brain dysfunction in sepsis related to the presence of bacteria at all?

Bacteremia is detected in only about a third of patients with sepsis, leading to the hypothesis that sterile mediators, such as LPS, or a dysregulated host response to infection are primary drivers of organ dysfunction (1, 35–38). Significant controversy surrounds the fidelity of sterile endotoxemia as a model of human sepsis, however (12, 39, 40). Our data suggest that viable bacteria do translocate to the brain during sepsis, even in the absence of overt infection. In murine sepsis survivors, cultivable aerobic bacteria are detected in the brain without bacteremia, suggesting that translocation either occurs very briefly or that the biomass of bacteria in blood is low, and bacteria are more readily detected once captured in the brain microvasculature. The identification of uncultured anaerobes in the brain, however, suggests that many disseminated bacteria may simply be undetectable with culture-dependent methods.

Given that the immune response to pathogen-associated molecules varies immensely with the chronicity, multiplicity, and intensity of exposure, understanding dissemination of even small numbers of bacteria is of critical importance to predicting and understanding the pathogenesis of organ dysfunction in this syndrome (14). Although we found that

Figure 5. Neuroinflammation is enhanced in postsepsis brains and correlated with changes in brain microbiota in experimental sepsis. *S100A8* (*S100* calcium binding protein A8) expression was correlated both with OTU008 abundance (A, permutational multivariate ANOVA [*adonis*], $P < 0.001$) and overall microbial community structure 5 days after operations (B, permutational multivariate ANOVA [*adonis*], $P \leq 0.001$). (B) Relative expression of *S100A8* is proportional to marker size. In addition to increased tissue damage-associated molecular pattern expression, sepsis survivor mice exhibit increased reactivity by microglia and brain tissue macrophages. (C) Immunofluorescence of motor cortex show markedly increased size and density of *Iba1*⁺ microglial cell processes. (D) Immunoreactivity for *Iba1* is increased in cecal ligation and puncture survivors on Day 5 post-cecal ligation and puncture in both area CA1 and overlying motor cortex (Mann-Whitney *U* test, $P = 0.035$ in both areas). $*P < 0.05$. (E) Microglia/macrophages were isolated by flow cytometry as a CX3CR1⁺CCR2⁺ cell population. (F) When stimulated *ex vivo* with LPS, CX3CR1⁺CCR2⁺ cells isolated from sepsis survivor mice 5 days post-cecal ligation and puncture produce increased tumor necrosis factor- α compared with sham-operated (ANOVA, $P < 0.0001$; Sidak *post hoc*, $P = 0.0002$) or antibiotic-treated mice (Sidak *post hoc*, $P < 0.0001$). $***P < 0.001$. Values are presented as means $\pm$ SEM. A.U. = arbitrary units; CCR2 = C-C motif chemokine receptor 2; CLP = cecal ligation and puncture; CX3CR1 = C-X3-C motif chemokine receptor 1; GFP = green fluorescent protein; *Iba1* = ionized calcium binding adapter molecule 1; OTU = operational taxonomic unit; PC = principal component; RFP = red fluorescent protein; *S100A8/A9* = *S100* calcium binding protein A8 and A9 heterodimer; TNF = tumor necrosis factor.

alterations in brain microbiota are associated with neuroinflammation, we do not claim that exposure to microbial pathogens is the sole pathway to developing acute or chronic brain dysfunction in critical illness. Delirium is associated with multiple causes of critical illness, and survivors of respiratory failure and shock suffer from chronic brain dysfunction (5, 41, 42). Microglia are activated in the brains of patients with delirium, even in the absence of sepsis (43). Multiple mechanisms, including ischemia, neurotransmitter imbalance, and vascular dysfunction likely contribute to brain dysfunction in critical illness (7). Nevertheless, there is evidence that amyloid fibrils, a pathologic hallmark of Alzheimer's disease, play a role in host defense against bacteria, suggesting immune mechanisms have evolved in response to direct exposure of the brain to bacteria (44). Even in primarily noninfectious states of critical illness, such as stroke, increases in gut permeability may increase risk of bacterial translocation, and modulation of the gut microbiota may be a target for treatment and reduction of complications (45).

The Bacteroidales OTU008 identified in our mouse studies is most closely homologous to the candidate family *Homeothermaceae*. Although the potential pathogenicity of these bacteria is unknown, they do carry genes for tolerance of oxidative stress and assembly of fimbriae, similar to pathogens in the genus *Porphyromonas* (46). A member of this family has recently been cultured for the first time, opening the possibility for cultivating other members of the *Homeothermaceae* and directly investigating their pathogenicity (47).

Although our results support the hypothesis that bacterial translocation is involved in the initiation of neuroinflammation, we did not find evidence of chronic bacterial infection of the brain in this model. In some forms of severe CLP, chronic intraabdominal infection is established (48, 49), but in our model peritoneal infection and inflammation were controlled. We did not find evidence of bacteria in the brain 2 weeks after sepsis, and thus persistent neuroinflammation after sepsis is likely caused by reprogramming of the host immune response and is not caused by persistent or chronic infection (9, 50, 51).

Both our animal and human studies demonstrate only an association, and not a causative relationship between brain microbial exposure and neuroinflammation. It is possible that differences in microbiota and neuroinflammation observed in the human and animal studies here are unrelated results of systemic inflammation in sepsis. A limitation of using CLP as a model of sepsis is that the gut serves as the primary source of infection. Antibiotic treatment to eliminate gut microbiota would alter the fundamental severity of illness in CLP, and therefore not illuminate the independent role of bacterial dissemination in neuroinflammation. We are developing other models of sepsis survival to address the independent contribution of the gut microbiota to long-term organ dysfunction after sepsis. Furthermore, although the use of the animal model allows us to study variation in bacterial taxa in the brain over time, the study of bacteria in the brains of patients with sepsis is necessarily limited by the requirement that patients die of their acute illness, which introduces several sources of confounding. The small number of patients in this study preclude adjusting for these factors, but they provide useful guidance for the design of future studies.

Several important factors other than brain microbiota differ among the sepsis and nonsepsis groups in our study. Patients in the sepsis group received more antibiotics than patients who died of nonsepsis causes. Although we postulate that the brain parenchyma is sterile in health, patient-derived samples are subject to translocation and outgrowth of bacteria during the postmortem interval (52–54). Administration of antibiotics during life could thus alter the 16S sequences observed in our study. The gut microbiome contributes to maintenance of the blood-brain barrier, cerebral leukocyte trafficking, and neurogenesis via circulating bacterial metabolites (55–57). Thus, antibiotic-induced dysbiosis could contribute to brain dysfunction even in the absence of sepsis.

Neurodegenerative disease was also more prevalent in the sepsis group than nonsepsis control subjects. Not all patients with elevated brain levels of S100A8/A9 had an underlying diagnosis of Alzheimer's disease. Alzheimer's disease is associated with increased levels of S100A8 and S100A9, although whether the fibrillar

forms of these proteins would react to the heterodimer-specific assay used here is unknown (58–60). As damage-associated molecular pattern, S100A8/A9 is also elevated by ischemia and hypoxia, not only in sepsis or infection (61–63). Patients in the sepsis group did have a prolonged agonal course compared with patients without sepsis, and signs of hypoxia have been identified previously in postmortem brain specimens from patients with sepsis and delirium (34). We unsuccessfully attempted to find alternative protein markers of brain inflammation in these tissues. The utility of S100A8/A9 as a marker of inflammation in this setting may be caused by high initial expression, or the robustness of this marker to the storage conditions in banked samples.

Despite these confounding factors, our human and murine data illuminate promises and pitfalls of using 16S sequencing to analyze brain microbiota. Although a previous study examined human brain microbiota using culture-independent techniques, it included patients with a heterogeneous group of infections and acute brain pathologies (64). Sequencing of 16S rRNA gene sequences in perfused brain tissue obtained under controlled conditions from antibiotic-treated control mice produces signals associated with multiple bacteria, including enteric pathogens, which are not present in reagent-only control animals. Several factors account for this discrepancy. First, microbial DNA may be present in the circulation or within circulating leukocytes in the absence of viable organisms. Nevertheless, we showed that the most abundant Bacteroidales 16S DNA detected in this study was derived from a live organism. Second, errors in both PCR amplification and gene sequencing are likely to be more frequent when abundant nucleic acids are present in a biologic sample than in reagent control subjects (65). It is unlikely that viable aerobic bacteria are consistently present within the healthy mouse brain with an abundance below the limit of detection by culture-based methods.

We have shown that sepsis results in altered brain bacterial taxa, which are associated with neuroinflammation in both a murine model of sepsis survival and in patients who die of sepsis, suggesting that direct translocation of bacteria to the brain plays a role in brain dysfunction in sepsis.

Currently, antimicrobial therapy in sepsis is often directed at the presumed source of infection, and frequently does not include empiric treatment of gut-associated anaerobic bacteria. Clinical trials have demonstrated that selective decontamination of gut bacteria reduces organ dysfunction and improves outcomes in sepsis, although the practice has not been adopted worldwide (66). This study and others suggest that

renewed attention should be paid to the effects of uncultured bacteria on specific end-organ dysfunctions in sepsis, and whether early antibiotic treatment of central nervous system infection even in the absence of encephalitis will modify the incidence of brain dysfunction after sepsis. Further study is needed, however, to delineate whether specific bacteria play a causative role in neuroinflammation. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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